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# Highly Efficient Protein Detection and Purification from Mammalian Cells Using HaloTag®...

Highly Efficient Protein Detection and Purification from Mammalian Cells Using HaloTag® Technology


## ABSTRACT

*Cultured mammalian cells are favored for producing functional mammalian proteins containing appropriate post translational modifications.Purification of recombinant proteins often is limited by low expression. The HaloTag® Mammalian Protein Detection and Purification Systems address this limitation by providing rapid detection and efficient protein purification regardless of expression level. This system uses the HaloTag® protein, a protein fusion tag that enables highly specific and covalent capture of a HaloTag® fusion protein of interest onto the HaloLink™ Resin. Efficient protein capture, coupled with proteolytic release of the protein of interest from the resin, results in superior recovery. The protein recovered is highly pure. In this study, we show that protein purification using the HaloTag® protein tag offers significant advantage over 3x FLAG® and $His_6$tag methods with respect to yield and purity.*



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### Introduction

Cultured mammalian cells offer an environment well suited for producing properly folded and functional mammalian proteins with appropriate post translational modifications(1) (2) . However, the low expression levels of recombinant proteins in cultured mammalian cells presents a challenge for their purification. As a result, attaining satisfactory yield and purity depends on selective and efficient capture of these proteins from the crude cell lysate.

Affinity tag-based chromatography is commonly used to facilitate protein purification. This technique uses specific and reversible interactions between the tagged protein of interest (i.e., fusion protein) and purification resin(3) (4) . The equilibrium-based binding means that the protein is constantly being exchanged between the bound (to the resin) and unbound state. Since this equilibrium depends on the protein concentration and the binding affinity of the tag at low expression levels, binding efficiency may be reduced, leading to low recovery of the fusion protein. In addition, the loss of bound protein during the purification process can further reduce yield.

The HaloTag® platform provides a new purification approach based on covalent capture that addresses these limitations(5) (6) . The highly specific and irreversible binding of the HaloTag® protein to the HaloLink™ Resin enables efficient protein capture regardless of the expression level and significantly reduces protein loss during washes of the resin, resulting in higher protein recovery. Since covalently bound fusion protein can't be eluted by conventional methods, the protein of interest is released from the resin by specific cleavage at an optimized TEV recognition site. In addition, the absence of any endogenous homologs of HaloTag® protein in mammalian cells minimize concerns regarding cross-reactivity and makes for a highly selective purification process. These features likely all contribute to the higher purity and yield we achieved in this study with the HaloTag® protein tag compared to 3xFLAG® and $His_6$tag systems. In addition, the ability of HaloTag® fusion protein to bind a series of customized ligands carrying different functional groups, including solid supports and fluorescent dyes, enables

a broad range of applications such as cellular imaging, cell sorting, protein detection, interaction studies and purification(5) (6) (7) (8) .

**Overview of the HaloTag® Mammalian Protein Detection and Purification System**

The HaloTag® Mammalian Protein Detection and Purification Systems utilize the HaloTag® protein tag, which can be genetically fused to any protein and transiently or stably expressed in mammalian cells. Alternatively, a collection of pre-cloned and experimentally-validated human ORFs fused to HaloTag is available through a partnership with Kazusa DNA Research Institute. Following cell lysis, the HaloTag® fusion protein is covalently captured on the HaloLink™ Resin, and nonspecific proteins are washed away. The protein of interest is then released by a specific proteolytic cleavage at an optimized TEV recognition site contained within the amino acid linker sequence that connects the HaloTag® protein tag and the protein of interest. The use of a TEV protease fused to HaloTag® (HaloTEV), which is covalently captured on the HaloLink™ Resin, eliminates the need for a secondary step to remove the protease, resulting in a streamlined purification process. This straightforward purification uses a single, mild physiological buffer throughout the entire process with no need for buffer exchange (Figure 1). In addition, the fluorescent HaloTag® TMRDirect™ Ligand provided with the system is useful for rapid protein labeling. The stability of the bond between HaloTag® protein and the fluorescent ligand allows for resolution by SDS-PAGE without loss of labeled protein, providing a simple and rapid alternative to antibody-based detection.

### Obtaining Highly Pure Tag-Free Protein of Interest

To demonstrate the benefits of the HaloTag® Mammalian Protein Detection and Purification Systems using cultured mammalian cells, we cloned p65 (RelA) into the HaloTag® Flexi® vector pFC14, and transiently expressed it as a C-terminal p65-HaloTag® fusion protein in HEK293T cells. The fluorescent detection of p65-HaloTag® and purification of the p65 protein (Figure 2) were performed as described in Technical Manual #TM348 (with modifications described in Figure 2). To determine the binding and purification efficiency, we resolved the following fractions by SDS-PAGE: soluble lysate and unbound fraction labeled with the HaloTag® TMRDirect™ Ligand as well as the purified protein (elution 1 and elution 2). The gel was scanned on a fluorescent scanner (Figure 2, Panel A) and we compared the fluorescence intensity of labeled HaloTag® fusion in the soluble lysates and unbound fractions and determined the binding efficiency. SimplyBlue staining (Figure 2, Panel B) demonstrated that the protein was highly efficient, pure and of the expected molecular weight.  These results indicate that the HaloTag® purification method provides an efficient protein capture mechanism and enables recovery of protein with little or no contaminates.

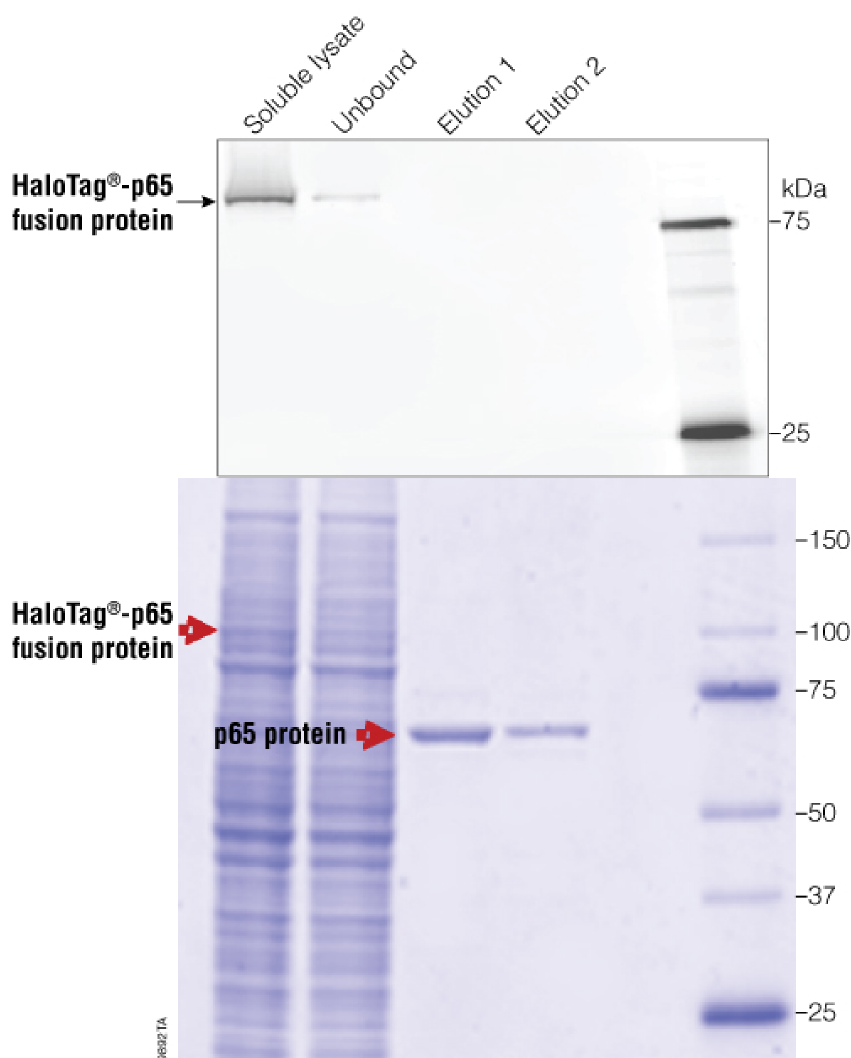


**Figure 2. Protein purification using the HaloTag® Mammalian Protein Purification method results in tag-free p65 protein.** A cell pellet from 2×10^{8} HEK293T cells was resuspended in 5ml of HaloTag® purification buffer (1X PBS, 1mM DTT, 0.005% IGEPAL® CA-630) supplemented with 1X Protease Inhibitor Cocktail, disrupted by sonication and harvested at 4°C (10,000 × g

for 15 minutes). The supernatant was added to 150µl of settled HaloLink™ Resin and incubated at room temperature for 1.5 hours with constant mixing. Following binding and three washes, the p65 protein was released by incubating the resin with 300µl of HaloTag® Purification Buffer supplemented with 45units of HaloTEV Protease at room temperature for 1.5 hours with constant mixing. The supernatant containing the released p65 was collected (Elution 1) and then the resin was washed with an additional 300µl of HaloTag® Purification Buffer for 30 minutes, and Elution 2 was collected. The soluble lysate and unbound fraction were labeled with 100nM HaloTag® TMRDirect Ligand for 30 minutes prior to SDS-PAGE analysis. **Panel A.** Fluorescent scan of the SDS-PAGE on a Typhoon® 9400 Instrument ($E_{ex}$=532nm, $E_{em}$=580nm). **Panel B.** SimplyBlue® stain of the same gel; arrows indicate HaloTag®-p65 fusion protein and the p65 protein alone, following cleavage.

### Highly Efficient Protein Recovery

Purification efficiency and purity are critical aspects to any purification method. We compared the HaloTag® Mammalian Purification method with two other commonly used affinity tags, the 3xFLAG® system (Sigma) and polyhistidine ($His_6$tag), for purity and percent yield of the expressed p65 protein (Figure 3). The p65 protein was successfully purified using all three methods; however, the protein yield and purity was significantly higher with the HaloTag® Mammalian Protein Purification and Labeling Systems (Figure 3, Panel A). This likely is due to the high selectivity of the HaloLink™ Resin, the absence of homologous endogenous proteins in the mammalian cells, and the ability to perform extensive washes with little to no loss of bound protein. The poor performance of the polyhistidine tag was most likely a result of the presence of histidine-rich endogenous proteins with high affinity to metal ions, which will bind to the HisLink™ Resin and co-purify with the $His_6$tag fusion protein.

To evaluate the efficiency of each purification method, we determined the fraction of the expressed protein that could be recovered using the different purification methods (Figure 3, Panel B). Using normalized volumes of soluble lysates, unbound fractions and purified proteins, we resolved the proteins by SDS-PAGE followed by Western blot analysis using a primary antibody against p65 and a secondary Cy®5-labeled antibody. Quantitation of the fluorescently scanned membrane indicated that 84% of the expressed protein could be recovered using the HaloTag® Mammalian Protein Detection and Purification System, compared to only 45% and 35% of the expressed protein recovered using 3xFLAG® and His6tag purifications, respectively. These results suggest that covalent immobilization provides more efficient protein capture and higher protein recovery.

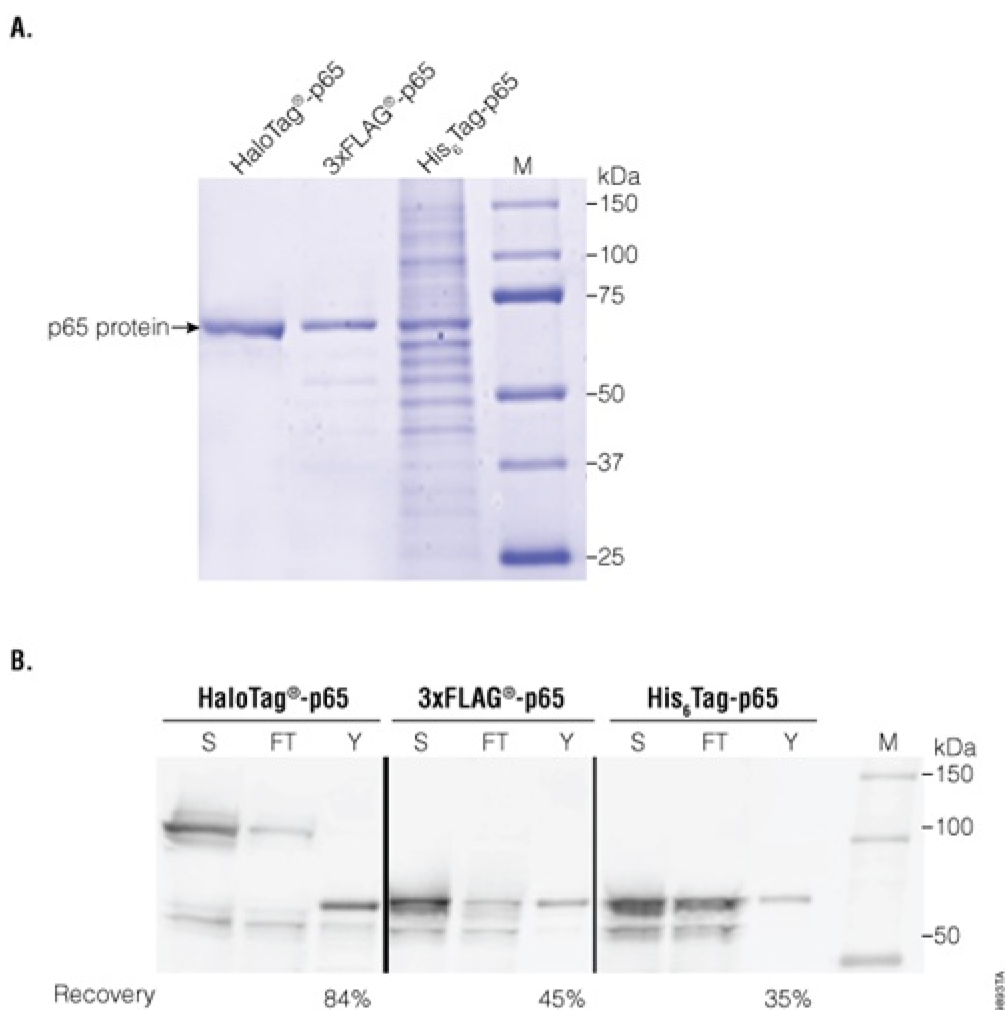


**Figure 3. Comparison of the HaloTag® Mammalian Purification System to the 3x FLAG and his6tag systems with regard to protein purity and yield.** HEK293T cells ($2 \times 10^8$) expressing the p65 protein with different C-terminal tags (HaloTag® Protein Tag, 3x FLAG® or his6tag) were harvested, and the appropriate systems were used to purify the p65 protein. The HaloTag® Mammalian Protein Purification was performed as described in Figure 2. The p65-3x FLAG® fusion protein was purified using Anti FLAG® agarose resin (Sigma) and eluted by low pH, as recommended by the manufacturer. The p65-his6tag fusion protein was purified using HisLink™ Resin (Promega) and eluted with 250mM imidazol. **Panel A.** Purified p65 protein was analyzed by SDS-PAGE and stained with SimplyBlue®. **Panel B.** Fluorescent scan of Western blot analysis using a primary antibody against p65 and a secondary Cy®5-labeled antibody. The scan was performed using a Typhoon® 9400 Instrument ($E_{ex}$=633nm, $E_{em}$=670nm) and bands were quantified using ImageQuant™ software.

## Highly Efficient Purification of Low-Abundant Protein

Occasionally it is not possible to over-express a protein of interest. At other times, protein expression levels might be lowered to reduce concerns regarding toxicity, or the capacity of the cell to produce mature protein and to incorporate the necessary post translational modifications.  For these reasons, we compared the ability of the HaloTag® Mammalian Protein Purification System, to that of the 3xFLAG® and $His_6$tag systems to purify low-abundant proteins. To reduce protein expression levels we replaced the CMV promoter region of the p65-fusion protein constructs with a truncated CMV promoter region that previously was shown to lower expression(9) . Western blot analysis in Figure 4 shows the expression of the p65 fusions under the control of a full CMV promoter (F) and the truncated CMV promoter (Δ). We observed a 90% reduction in expression level of the fusion proteins when using the truncated CMV promoter.

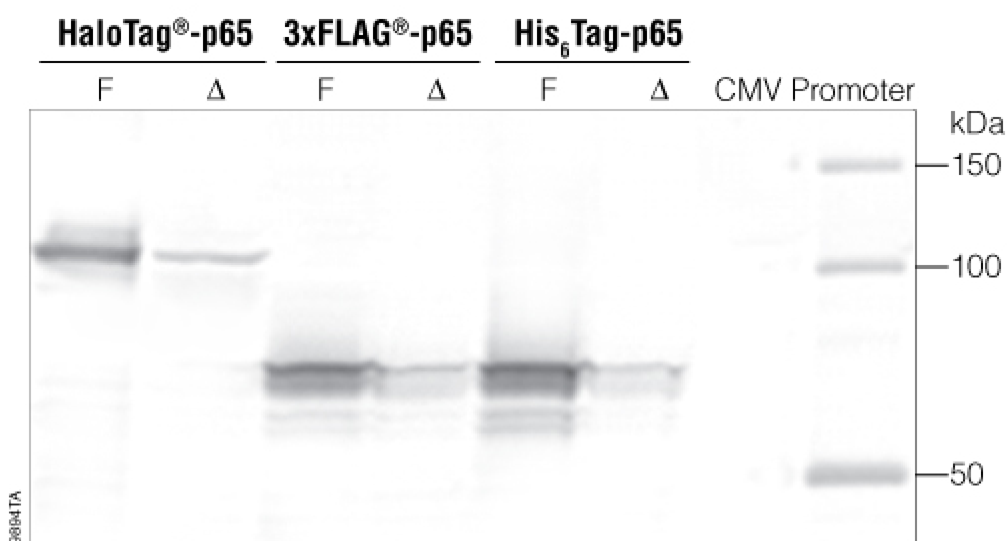


**Figure 4. Comparison of expression levels under the control of a full or truncated CMV promoter and yield.** Lysates from $2 \times 10^8$ HEK293T cells expressing C-terminal p65 fusions under the control of a full or truncated CMV promoter were analyzed by Western blot analysis as described in Figure 3.

To compare the purification methods for purity and total yield of the expressed protein, the p65 fusion proteins were transiently expressed under the control of the truncated CMV promoter and purified (Figure 5).  Again, the HaloTag® System was more efficient as indicated by the higher purity and overall yield.  The Western blot analysis shown in Figure 5, Panel B, indicates that, although the expression level was reduced to 10% of that seen with the full CMV promoter, HaloTag® System recovered 74% of the expressed protein, compared to only 27% and 9% using 3xFLAG® and $His_6$tag purifications, respectively. These results indicate that covalent protein capture is less affected by expression levels, resulting in efficient purification even when the amount of fusion protein is low.

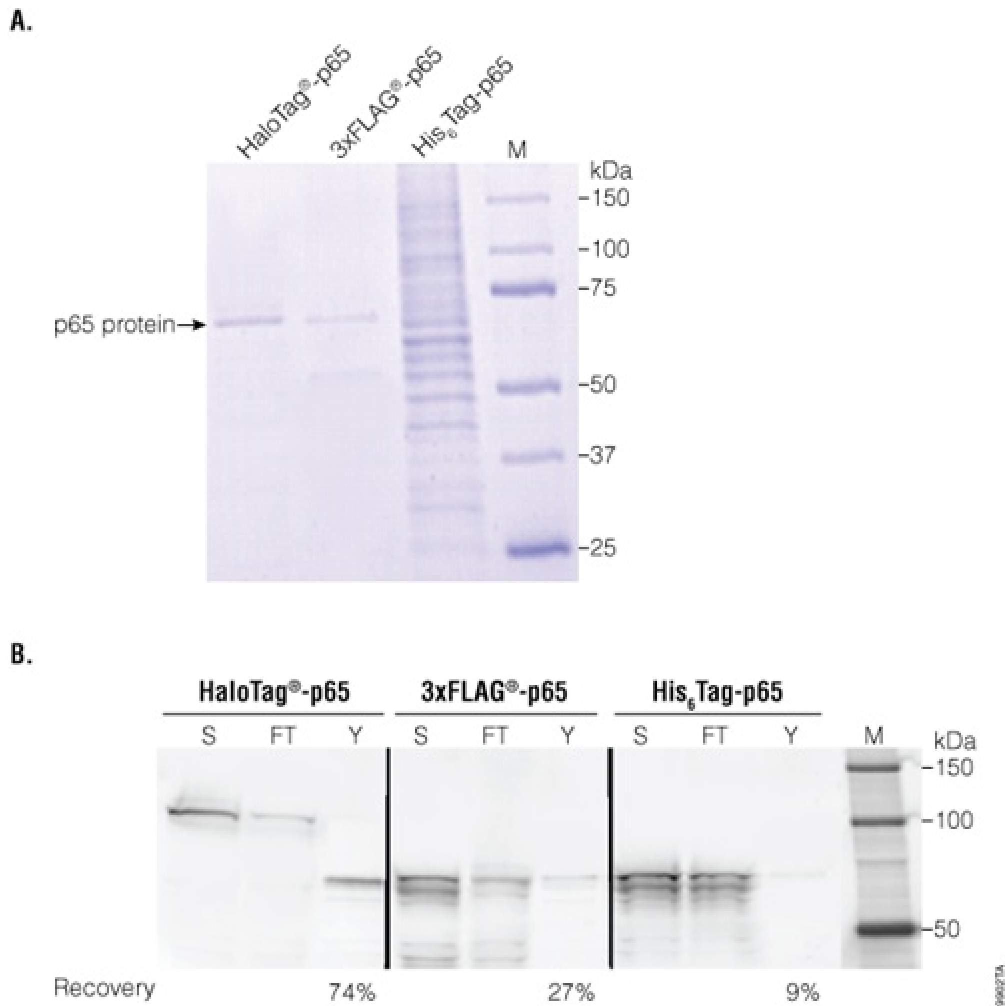


**Figure 5. Comparison of C-terminal tags for purification of low abundant protein.** HEK293T cells (2 × $10^8$ ) expressing the p65 protein with different C-terminal tags (HaloTag® Protein, 3x FLAG® or $His_6$Tag under the control of a truncated CMV promoter) were harvested and the p65 protein was purified as described in Figure 3. **Panel A.** Purified p65 protein analyzed by SDS-PAGE and stained with SimplyBlue®. **Panel B.** Western blot analysis of normalized volumes of soluble lysates, unbound fractions and purified using a primary antibody against p65 and a secondary Cy®5-labeled antibody. The membrane was scanned on a Typhoon® 9400 Instrument ($E_{ex}$=633nm, $E_{em}$=670nm), and bands were quantified using ImageQuant™ software.

## Conclusions

The results from this study and another comparing the purification of five human kinases [data not shown;(5) ] indicate that HaloTag® Mammalian Protein Purification and Labeling Systems outperform the traditional affinity tags, $His_6$tag and 3xFLAG®, with respect to yield, purity and percent recovery of the expressed proteins. These results were even more pronounced in the purification of protein expressed at low levels. The HaloLink™ Resin has the combined advantage of good binding capacity (≥7mg/ml, 10X more capacity than 3xFLAG® Resin) and a high degree of specificity and selectivity because of the absence of analogous endogenous proteins. In addition, covalent protein capture is not dependant on protein expression levels and allows for extensive washes without loss of bound proteins. These features differentiate the HaloTag® protein tag from other affinity tags and provide a considerable advantage over existing approaches.

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